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Role of Oxidative Stress in the Radiation-Induced Lung Pathogenesis in Mice

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In pre-transplant total-body irradiation (TBI), the lung is a critical dose-limiting organ. Also, the possible role of oxidative stress was suggested in the development of TBI-induced lung damage. This study explores the association between TBI-induced oxidative stress and the induction of lung pathogenesis by investigating TBI-induced oxidative stress in the lungs of male C57BL/6 mice after a single dose of 10 Gy TBI. We showed significant increases of reactive oxygen species (ROS) formation and lipid peroxidation, and also a depletion and oxidation of glutathione after TBI. There is evidence that pretreatment with 1,10-phenanthroline (*o*-phen) significantly reduces oxidative stress in the lung. This indicates that the TBI-induced ROS generation involves a metal-catalyzed Fenton-type reaction. A pretreatment of buthionine sulfoximine (BSO) augmented the glutathione depletion and oxidation, but had no effect on the ROS formation and lipid peroxidation up to 6 h after TBI. Histopathological features that are consistent with pneumonitis were observed in the BSO pretreated-mice 1 week after irradiation. The results suggest that TBI-induced oxidative stress in the lung involves a generation of ROS through a Fenton-type reaction. Also, glutathione plays an important inhibitory role in the radiation-induced lung pathogenesis by participating in the self-amplifying cascade subsequent to the ROS generation by irradiation.

Keywords: Glutathione, Lung, Oxidative stress, Radiation

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Introduction

Bone marrow transplantation (BMT) is now a standard treatment for leukemia and other hematological malignancies (Krowka *et al.*, 1985; Weiner *et al.*, 1986; Wingard *et al.*, 1988). The lung is known as a dose-limiting organ for total-body irradiation (TBI) in preparatory TBI. This is primarily due to the higher O_2 levels than in other tissues. The damaging effect of ionizing radiation is aggravated by the presence of dissolved oxygen (Revesz and Palcic, 1985). A high-dose pre-transplant TBI regimen predisposes 10-50% of the patients to lung complication with a fatality rate of 20-80% (Barrett *et al.*, 1983; Tesghma *et al.*, 1986; Latini *et al.*, 1992). Where irradiation of the thoracic region is involved, the lung also represents a dose-limiting organ (Hall *et al.*, 1988; Coset *et al.*, 1989; Kraus *et al.*, 1991).

Despite substantial research efforts (Steinberg *et al.*, 1992; Steinberg *et al.*, 1993; De *et al.*, 1995), the nature of the radiation-induced early events that link to the late lung pathogenesis (such as inflammation, pneumonitis and fibrosis) remains obscure. The possible role of oxidative stress in TBI-induced lung pathogenesis has been suggested since biological consequences of ionizing radiation involve various transient reactive oxygen species (ROS), such as superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\bullet}$) (Levinson, 1966; Walburg, 1975; Simic *et al.*, 1989). A generation of ROS by the interaction of ionizing radiation with water molecules is well recognized. Oxidative stress that is induced by ROS can damage important biomolecules (Koh *et al.*, 1999; Cho and Song, 2000) in the lung that could eventually lead to lung pathogenesis. There is little experimental data, however, to support this view. This study explores the association between TBI-induced oxidative stress and the induction of lung pathogenesis. We analyzed the lungs of C57BL/6 mice for the ROS formation, and assessed oxidative damage to the lung by measuring lipid peroxidation

and glutathione changes at 0.1, 2, and 6 h after irradiation. We know that glutathione (γ-glutamylcysteinylglycine) *in vivo* is predominantly present as a reduced (GSH) form under normal physiological conditions. Under oxidative stress conditions, GSH is oxidized to GSSG, which in turn can be exported out, or recycled to GSH (Sies, 1985; Thom *et al.*, 1997). Therefore, the cellular content of total glutathione (GSH and GSSG), as well as its redox change, is recognized as a sensitive indicator of oxidative stress. In addition, we examined the effect of the pretreatment of 1,10-phenanthroline (*o*-phen), a metal chelator, and buthionine sulfoximine (BSO), a γ-glutamylcysteine synthetase inhibitor (Griffith, 1982), to the TBI-induced oxidative damage to the lung. The histopathological features of the lungs of irradiated mice were analyzed 1 week after irradiation in order to assess the consequences of TBI-induced oxidative stress.

Materials and Methods

Materials Glutathione reductase, reduced glutathione (GSH), oxidized glutathione (GSSG), N-ethylmaleimide (NEM), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), NADPH, 2,4-dinitrofluorobenzene (FDNB), 2-thiobarbituric acid (TBA), 1,1,3,3-tetraethoxypropane (TEP), sodium dodecyl sulfate (SDS), 2',7'-dichlorofluorescin diacetate (DCFH-DA), 2',7'-dichlorofluorescein (DCF), ascorbic acid, $FeSO_4$, L-buthionine sulfoximine (BSO), and 1,10-phenanthroline (*o*-phen) were purchased from Sigma (St. Louis, MO, USA). The Spherisorb NH_2 column (particle size 5 μm, 25 cm × 4.6 mm) was from ISCO (Lincoln, NE, USA). All of the reagents were of analytical grade, or of the highest grade available. The protein assay reagent was from Bio-Rad (Hercules, CA, USA).

Animals and irradiation Unanesthetized male C57BL/6 mice (4 weeks old, weighing 20-30 g) were irradiated (10 Gy single fraction) with 4 MV X-ray (Siemens, MeVatron). An irradiation dose was calculated at the mid depth of mice in the field size of 40 × 40 cm with a dose rate of 0.2 Gy/min. Buthionine sulfoximine (BSO) (2 mmol/kg) and *o*-phen (15 mg/kg) were dissolved in a phosphate-buffered saline (PBS), and injected intraperitoneally 3 h and 30 min prior to irradiation, respectively. The control mice received PBS only, otherwise they were treated identically. For measurement of the ROS formation, lipid peroxidation, and glutathione, the animals were sacrificed by decapitation 0.1 h, 2 h, or 6 h after TBI. The organs were removed, washed, and frozen by freeze-clamping with dry ice-cooled tongs immediately after sacrifice in order to quench the metabolic reactions. The tissues were stored at −70°C until analyzed. For the histopathological analysis, the mice were returned to their cages after TBI. The mice were fed a standard laboratory diet and water *ad libitum*. They were kept for 1 week under a 12-h light/dark cycle until analyzed.

Measurement of glutathione Glutathione in the lung was analyzed by HPLC using a modified method of Reed *et al.* (1980). Briefly, 5% perchloric acid extract of the lung was derivatized with FDNB. Reduced (GSH) and oxidized glutathione (GSSG) were separated by HPLC on a Spherisorb NH_2 column. To avoid the effect of artifactual oxidation of glutathione during treatment, an extract with acid that contained 50 mM NEM was used to determine GSSG (Park *et al.*, 1998; Lee *et al.*, 1999). Total glutathione was expressed as the GSH equivalent to the sum of GSH and GSSG, that is, GSH + 2 GSSG. Glutathione was also analyzed by the enzymatic recycling method of Tietze (1969).

Measurement of reactive oxygen species (ROS) formation The rate of ROS formation in the lung extract was analyzed by using the probe DCFH-DA. DCFH is oxidized to fluorescent dichlorofluorescein (DCF) by reaction with ROS (LeBel *et al.*, 1992). The rate of ROS formation was calculated from the slope of fluorescence increase at the excitation wavelength of 485 nm and emission wavelength of 530 nm. This was calibrated with authentic DCF.

Measurement of lipid peroxidation The extent of lipid peroxidation in the lung was assessed by the method of Ohkawa *et al.* (1979) that measures the thiobarbituric acid-reactive substances (TBARS) in the tissue homogenate. The thiobarbituric acid-malondialdehyde adduct was measured by a fluorescence measurement at an excitation wavelength of 515 nm and emission wavelength of 553 nm. This was calibrated with 1,1,3,3-tetraethoxypropane.

Pathological analysis The lungs were fixed in 4% formaldehyde with a 0.1 M sodium phosphate buffer, pH 7.4. For microscopy, the specimen was dehydrated with ethanol and embedded in paraffin. The sectioned specimen was then stained with eosin and hematoxylin.

Statistical analysis The significance was calculated from means with the aid of a student's t-test. A determination of standard deviation resulted from a random test. Values are given as means ± standard deviations of the data obtained from at least 5 animals. P values of <0.05 were considered as significant, and indicated by asterisks in the figures.

Results

Induction of reactive oxygen species (ROS) formation and lipid peroxidation in the lung by total body irradiation To determine whether total body irradiation (TBI) induces oxidative stress in the lung, we first analyzed the extent of the reactive oxygen species (ROS) formation in the lungs from mice that received TBI at a single dose of 10 Gy. We quantitated the ROS formation in the lungs of irradiated mice by measuring fluorescence from the dichlorofluorescein (DCF) that was produced. Fig. 1 shows the result from the DCF assay of the lungs of the irradiated mice, followed by the calibration with authentic DCF. Our results revealed that TBI induced instantaneous increases in the ROS formation. This was maintained up to 6 h after irradiation. Reduction of the ROS formation was evident 6 h after the irradiation.

The extent of lipid peroxidation in the lung was determined by measuring 2-thiobarbituric acid-reactive substances (TBARS). Fig. 2 shows the temporal changes of lipid peroxidation in the lungs of irradiated mice. Our results

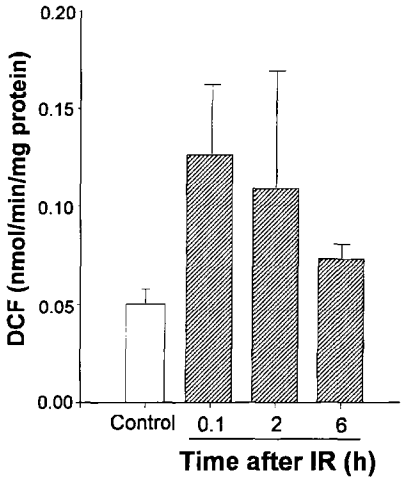

Fig. 1. TBI-induced changes in the rate of reactive oxygen species (ROS) formation. Mice were exposed to total-body irradiation (TBI) at a single dose of 10 Gy. Irradiated mice were sacrificed 0.1 h, 2 h, or 6 h after irradiation. The lungs were dissected and analyzed for ROS formation by using the probe DCFH-DA. Values are expressed as the rate of DCF formation per mg protein (nmol/min/mg protein). Results are expressed as means ± standard deviations (n=5).

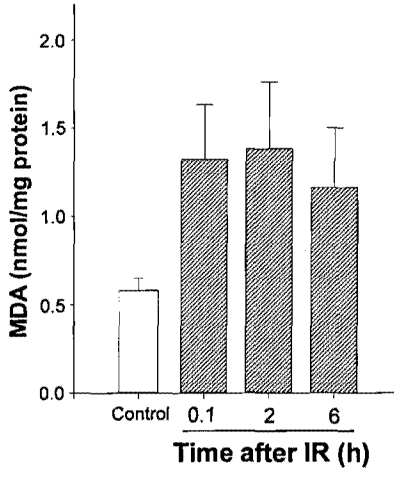

Fig. 2. TBI-induced changes in lipid peroxidation. The lungs of the irradiated (single dose, 10 Gy) mice (Fig. 1) were analyzed for lipid peroxidation by the method that measures thiobarbituric acid-reactive substances (TBARS) in the tissue homogenate. Values are expressed as nmol MDA/mg protein. Results are expressed as means ± standard deviations (n=5).

revealed significant increases of lipid peroxidation after TBI, which demonstrates a reasonably good correlation with the extent of the ROS formation.

Change of the lung glutathione status by total body irradiation The cellular content of total glutathione (GSH and GSSG), as well as its redox change, is recognized as a sensitive indicator of oxidative stress. We analyzed therefore the change of glutathione status in the irradiated lungs.

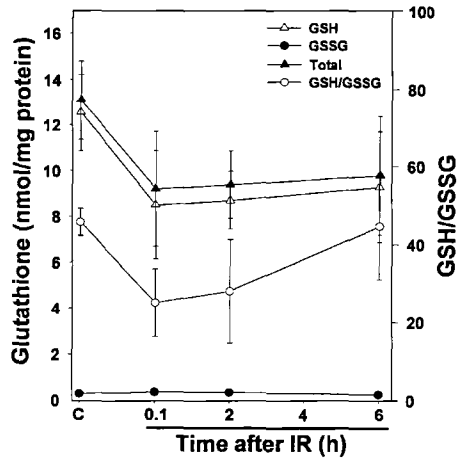

Fig. 3. TBI-induced changes in glutathione. The lungs of the irradiated (single dose, 10 Gy) mice (Fig. 1) were analyzed for glutathione. The acid extracts of the lung were derivatized with FDNB. Reduced (GSH) and oxidized glutathione (GSSG) in the samples were separated and analyzed by the HPLC method. Total glutathione was expressed as the GSH equivalent to the sum of GSH and GSSG. Results are expressed as means ± standard deviations (n=5).

Our results revealed approximately a 30% reduction in total glutathione content (from 13.08 ± 1.74 to 9.21 ± 2.50) almost immediately after irradiation (Fig. 3). At the same time, the relative ratio of GSH/GSSG was reduced from 45.5 9± 3.47 to 24.97 ± 8.69. The amount of total glutathione then increased slowly. Although the relative ratio of GSH/GSSG returned almost to the control value 6 h after irradiation, the amount of total glutathione (9.81 ± 2.58) was consistently lower than the control value. It is likely that since the amount of GSH remained consistently low up to 6 h after irradiation, the glutathione redox balance recovered in favor of the reduced state at the expense of the GSSG efflux.

Inhibition of TBI-induced oxidative damage in the lung by pretreatment with 1,10-phenanthroline (*o*-phen) We then examined the effect of *o*-phen, a metal chelator, on the TBI-induced oxidative stress in the lung. As shown in Figs. 4 and 5, pretreatment with *o*-phen completely prevented the TBI-induced ROS formation and lipid peroxidation. These results strongly suggest that TBI-induced oxidative stress involves a generation of ROS through a metal catalyzed Fenton-type reaction (Sutton and Winterbourn, 1989; Halliwell and Gutteridge, 1992).

We noted a minor reduction in the steady state levels of the ROS formation in mice that were pretreated with *o*-phen without the TBI exposure. This indicates that *o*-phen reduced a generation of the steady-state level of ROS.

Augmentation of TBI-induced oxidative stress in the lung by pretreatment with buthionine sulfoximine (BSO) In order to examine the effect of glutathione on TBI-induced

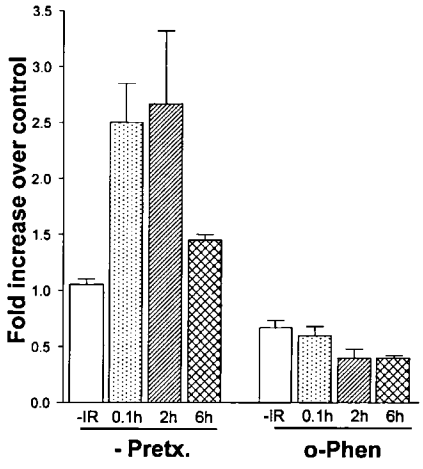

Fig. 4. Effect of 1,10-phenanthroline (*o*-phen)-pretreatment on TBI-induced reactive oxygen species (ROS) formation. Mice were injected with *o*-phen (15 mg/kg) intraperitoneally 30 min prior to irradiation (single dose, 10 Gy). Mice were sacrificed 0.1 h, 2 h, or 6 h after irradiation. The lungs of the mice were analyzed for ROS formation by using the probe DCFH-DA. Results are expressed as means ± standard deviations (n=5).

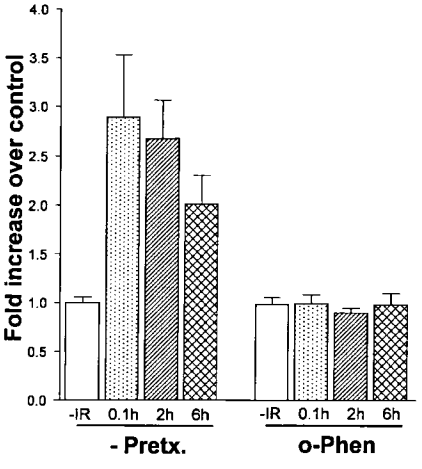

Fig. 5. Effect of 1,10-phenanthroline (*o*-phen)-pretreatment on TBI-induced lipid peroxidation. The lungs of the mice that were treated with *o*-phen and/or irradiation (Fig. 4) were analyzed for lipid peroxidation by the method that measures thiobarbituric acid-reactive substances (TBARS) in the tissue homogenate. Values are expressed as fold increase over control. Results are expressed as means ± standard deviations (n=5).

oxidative stress to the lung, we pretreated mice with BSO, an inhibitor of γ-glutamylcysteine synthetase (γ-GCS), 3 h prior to irradiation. We found a significant and consistent reduction of tissue glutathione following irradiation in BSO-pretreated mice (Fig. 6). In contrast to the mice that received TBI only, BSO-pretreated mice showed neither recovery of the total glutathione pool, nor a GSH/GSSG ratio up to 6 h after TBI. At 6 h after TBI, the total glutathione content was about 43% of the control value (control, 12.46 ± 1.30; 6 h, 5.37 ± 2.22), and the ratio of GSH/GSSG was about 50% of the control

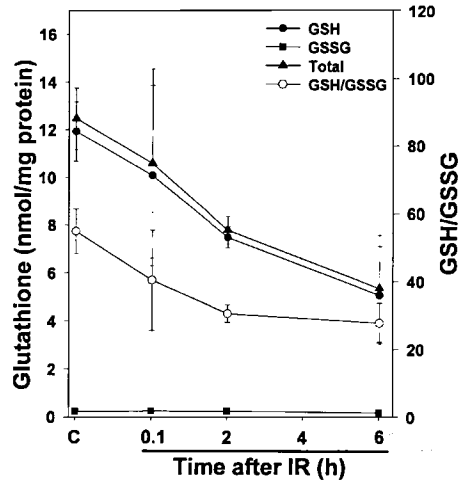

Fig. 6. Effect of buthionine sulfoximine (BSO)-pretreatment on TBI-induced changes in glutathione. Mice were injected with BSO (2 mmol/kg) intraperitoneally 3 h prior to irradiation (single dose, 10 Gy). Mice were sacrificed 0.1 h, 2 h, or 6 h after irradiation. The lungs were analyzed for glutathione. The acid extracts of the lung were derivatized with FDNB. Reduced (GSH) and oxidized glutathione (GSSG) in the samples were separated and analyzed by the HPLC method. Total glutathione was expressed as the GSH equivalent to the sum of GSH and GSSG. Results are expressed as means ± standard deviations (n=5).

(control, 54.6 ± 6.62; 6 h 27.8 ± 5.84). The BSO-pretreatment alone did not alter the levels of total glutathione (12.46 ± 1.30 with respect to 13.08 ± 1.74 without BSO-pretreatment), or the relative ratio of GSH/GSSG (45.59 ± 3.47 with respect to 45.28 ± 6.62) significantly.

To examine the histological features of the lung, mice were sacrificed 1 week after irradiation. As shown in Fig. 7, a histopathological examination of the lungs of the BSO-pretreated mice revealed inflammation, alveolar hemorrhage, widening, and destruction of the interalveolar septae that was filled with red cells (panel C). At the same post-irradiation time interval of 1 week, some widening and destruction of the interalveolar septae were observed in mice that only received TBI (panel B). A normal lung is shown for comparison (panel A).

Discussion

Although bone marrow transplantation (BMT) is often a standard treatment for leukemia and other hematological malignancies, the preparatory total body irradiation (TBI) that is undertaken prior to BMT predisposes the patients to lung complication at a significant fatality rate. In this study, we provide experimental evidence that the TBI-induced lung damage involves a generation of reactive oxygen species (ROS) by measuring the rate of the ROS formation and lipid peroxidation in the lungs of irradiated mice. We also demonstrate that tissue glutathione plays a key role in modulating TBI-induced lung pathogenesis.

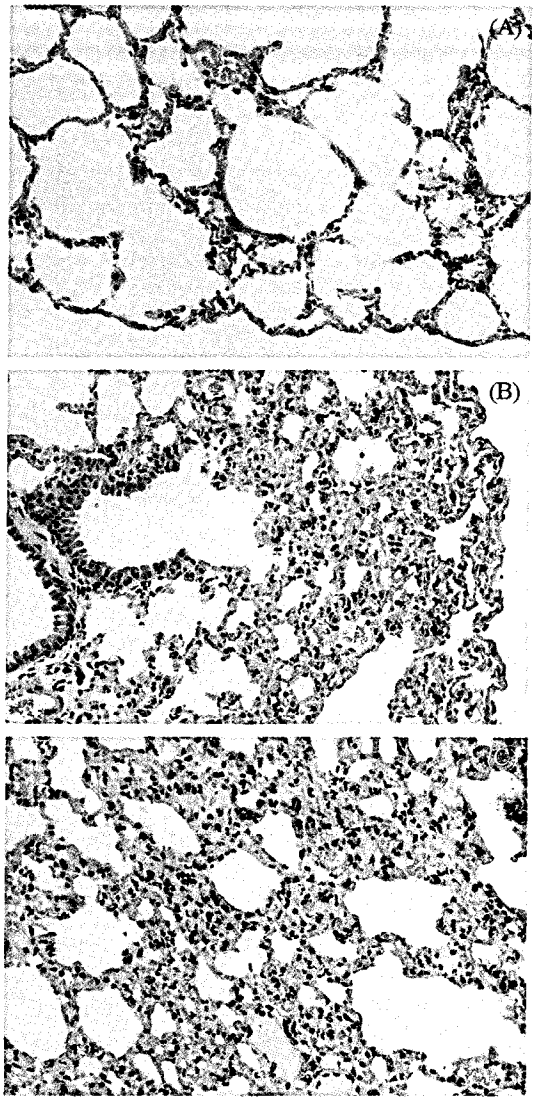

Fig. 7. Histopathological examination of the lungs of irradiated mice. The lungs of the control (panel A), TBI only-treated (panel B), and BSO and TBI-treated (panel C) mice were fixed, dehydrated, and embedded in paraffin. The sectioned specimens were then stained with eosin and hematoxylin.

Ionizing radiation interacts with water to form its ionization and excitation products, which is aggravated by the presence of dissolved oxygen (Revesz and Palcic, 1985). Therefore, the lung is presumably a prime target for radiation-induced damage, because it is exposed to higher oxygen levels than other tissues. Since water composes 55-80% of the mass of biological systems, the primary interaction of radiation in biological systems is with water in order to form various ROS. Our results show that TBI induces an almost instantaneous increase in the ROS formation in the lungs, then the amounts gradually decrease within a few hours. We assessed the TBI-induced oxidative damage to the lung by quantitating lipid peroxidation, since membrane lipids are particularly sensitive to ROS attack due to their unsaturated fatty acids.

Glutathione is an almost universal constituent of biological systems. It is recognized for its protective role against oxidative stress. Cellular glutathione (GSH) is oxidized to GSSG and the oxidized glutathione, GSSG, is either reduced by glutathione reductase or depleted from cells (Sies, 1986; Deneke and Fanburg, 1989). Therefore, glutathione can also serve as an indicator of oxidative stress. We show that TBI induced an almost instantaneous increase in lipid peroxidation, and decreases in total glutathione and GSH/GSSG in the lung. The degree of lipid peroxidation and glutathione depletion was maintained for several hours. On the other hand, the ratio of GSH/GSSG fully recovered 6 h after TBI. Based on the consistent low level of GSSG that was observed even after irradiation, the results can be interpreted as the following: ROS that is generated by irradiation induced the oxidation of GSH to GSSG, and GSSG was then exported from cells in order to maintain the reduced glutathione status in the lung. Therefore, the observed recovery of the glutathione reduction state appears to be primarily due to the depletion of GSSG from cells, rather than the reduction to GSH.

We attempted to investigate the effect of the pretreatment of mice with reagents that are capable of manipulating the degree of oxidative stress prior to TBI. 1,10-phenanthroline (*o*-phen) is a metal chelator that is known to have a good transport potential in cells (Mello Filho *et al.*, 1984). The *o*-phen-pretreatment completely prevented TBI-induced increases in the ROS formation and lipid peroxidation. The results strongly suggest that the TBI-induced ROS formation largely depends on a metal-catalyzed Fenton-type reaction. It is also responsible for the lipid peroxidation. Pretreatment with an inhibitor of γ-glutamylcysteine synthetase (γ-GCS), buthionine sulfoximine (BSO), exerted a significant effect on the glutathione status in the mice that received TBI. We found the phase of recovery from the TBI-induced glutathione depletion and oxidation (i.e., decrease in GSH/GSSG) in the mice that received TBI only. However, the recovery phase was absent in the mice that were pretreated with BSO, indicating that the glutathione pool was continuously depleted and oxidized. The results suggest that *de novo* synthesis of glutathione is active in the lung, and responsible for the recovery of the glutathione status in the lung that is under oxidative stress.

The BSO-pretreatment, however, did not augment the TBI-induced lipid peroxidation and ROS formation. We suspect the role of other antioxidant systems in the relatively short-term effect of TBI, such as the ROS formation and lipid peroxidation. On the other hand, a histological examination showed that the BSO-pretreatment clearly augmented TBI-induced lung damage at the post-irradiation time interval of 1 week. The result suggests that the inhibition of glutathione *de novo* synthesis greatly potentiates the later onset of lung pathogenesis. It also implicates a subsequent self-amplification of oxidative stress that is generated by TBI. However, we cannot rule out the possibility that the systemic

effect of TBI, such as the suppression of the immune system, may have a role in the underlying pathology in the lung.

The observed close correlation between the increased oxidative stress and pathogenesis in the lung provides experimental evidence that a generation of TBI-induced ROS is likely to be an important event that leads to the later onset of lung pathogenesis. We suspect that the generation of TBI-induced oxidative stress causes lung complications both directly, via causing oxidative damage to sensitive functional cells, and indirectly via influencing the systemic effect of preparatory TBI. Our data suggest the possibility of reducing the TBI-induced lung pathogenesis by proper antioxidant therapy.

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